Accepted Manuscript

Rapid Concentration and Sensitive Detection of Hookworm Ova from Wastewater Matrices Using a Real-Time PCR Method

P. Gyawali, J.P.S. Sidhu, W. Ahmed, P. Jagals, S. Toze

PII: S0014-4894(15)30026-6

DOI: 10.1016/j.exppara.2015.08.009

Reference: YEXPR 7112

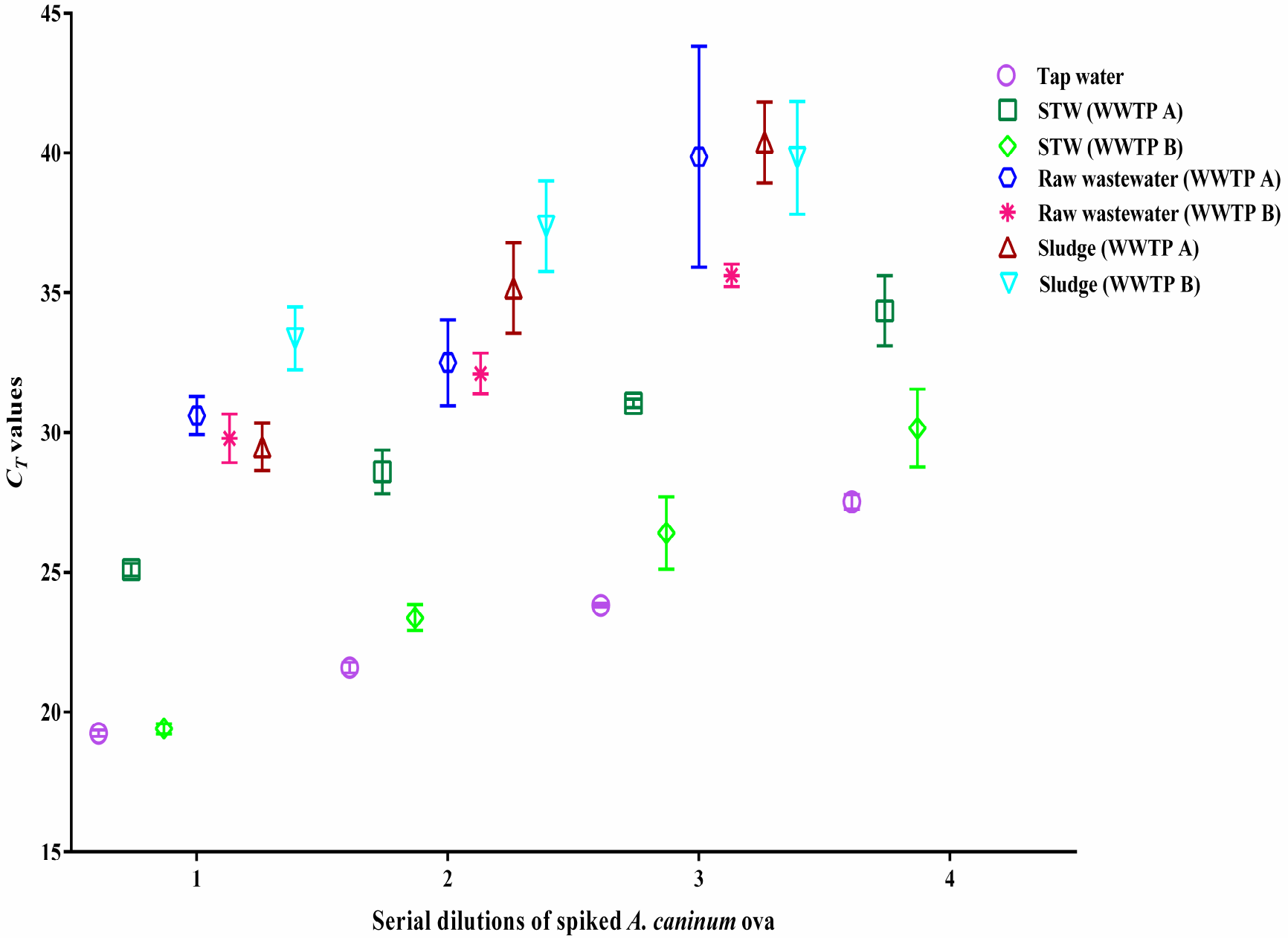
To appear in: *Experimental Parasitology*

Received Date: 8 March 2015

Revised Date: 31 July 2015

Accepted Date: 16 August 2015

Please cite this article as: Gyawali, P, Sidhu, J., Ahmed, W, Jagals, P, Toze, S, , Rapid Concentration and Sensitive Detection of Hookworm Ova from Wastewater Matrices Using a Real-Time PCR Method, *Experimental Parasitology* (2015), doi: 10.1016/j.exppara.2015.08.009.



Rapid Concentration and Sensitive Detection of Hookworm Ova from Wastewater Matrices Using a Real-Time PCR Method

P Gyawali[1, 2*], JPS Sidhu[1, 2], W Ahmed[1, 3], P Jagals[2] and S Toze[1, 2]

[1]CSIRO Land and Water, Ecosciences Precinct, 41 Boggo Road, Qld 4102, Australia, [2]School of Population Health, The University of Queensland, Herston Road, Qld 4006, Australia, [3]Faculty of Science, Health and Education, University of the Sunshine Coast, Maroochydore, DC, Qld 4558, Australia

Running title: Rapid detection of hookworm ova in wastewater matrices

*Correspondence: CSIRO Land and Water, Ecosciences Precinct, GPO Box 2583 Brisbane, QLD 4001 Australia, Tel: +61401 302 264, E- mail: pradep033@yahoo.com

Abstract

The risk of human hookworm infections from land application of wastewater matrices could be high in regions with high hookworm prevalence. A rapid, sensitive and specific hookworm detection method from wastewater matrices is required in order to assess human health risks. Currently available methods used to identify hookworm ova to the species level are time consuming and lack accuracy. In this study, a real-time PCR method was developed for the rapid, sensitive and specific detection of canine hookworm (*Ancylostoma caninum)* ova from wastewater matrices. *A. caninum* was chosen because of its morphological similarity to the human hookworm (*Ancylostoma duodenale* and *Necator americanus*). The newly developed PCR method has low detection sensitivity with the ability to detect less than one *A. caninum* ova from 1 L of secondary treated wastewater at the mean threshold cycle (C_T) values ranging from 30.1-34.3. The method is also able to detect four *A. caninum* ova from 1 L of raw wastewater and from ~4 gm of treated sludge with mean C_T values ranging from 35.6-39.8 and 39.8-39.9, respectively. The better detection sensitivity obtained for secondary treated wastewater compared to raw wastewater and sludge samples could be attributed to sample turbidity. The proposed method appears to be rapid, sensitive and specific compared to traditional methods and has potential to aid in the public health risk assessment associated with land application of wastewater matrices. Furthermore, the method can be adapted to detect other helminth ova of interest from wastewater matrices.

Keywords: Real-time PCR, Helminth, Hookworm, Wastewater, Sludge, Health Risk Assessment

1. Introduction

Hookworm infections in humans are a leading cause of malnutrition, anemia, physical and mental retardation in developing countries (Hotez et al., 2005; Brooker et al., 2008; WHO, 2012). *Ancylostoma duodenale* and *Necator americanus* are the primary cause of human infections (Hotez et al., 2005; Traub et al., 2008; WHO, 2012). Hookworm infections account for approximately 3.2×10^6 estimated disability adjusted life years (DALYs) as reported in the global disease burden study in 2010 (Hotez et al., 2014). Most of these infections are severe and can account for up to 1.4×10^5 deaths (Bethony et al., 2006; Knopp et al., 2012).

An adult hookworm can survive 5-7 years in their human host and produces 1.0×10^4-3.0×10^5 ova/day (Bethony et al., 2006). Hookworm ova are released into wastewater streams mainly through faeces of infected hosts (Toze and Sidhu, 2011; Gyawali, 2012). In the receiving environment, the ova can remain dormant for 9-12 months and can hatch into larvae under favourable conditions (Abaidoo et al. 2010). The infective larvae (L_3) can survive up to three months in the environment (Brooker et al., 2006; Brooker, 2010). This has implications for the use and reuse of human wastewater matrices in the agriculture (WHO, 2006; Karkashan et al., 2014).

In recent years, the use and reuse of wastewater matrices in broadacre agriculture have increased significantly worldwide (Carr, 2005; Sidhu and Toze, 2009; Pritchard et al., 2010; Hanjra et al., 2012). It has been estimated that 5.0×10^6 ha of agricultural lands are irrigated with raw wastewater (Carr, 2005), and up to 42% of treated sludge produced globally from wastewater treatment process is being used as fertiliser (Kelessidis and Stasinakis, 2012). Since a single viable ovum has the potential to cause an infections in an exposed humans, the health risks can be high for people handling wastewater matrices in areas where helminth infections are endemic especially developing countries (Ensink et al., 2005; WHO, 2006).

Currently used detection methods are not highly sensitive and specific to detect viable hookworm ova in wastewater matrices. Incubation and stain-based methods are widely used to detect hookworm ova in wastewater matrices (US EPA, 2003; de Victorica and Galván, 2003; Bowman et al., 2003 Do et al., 2007; Wen et al., 2009; Sharafi et al., 2012). One major limitation of the incubation method is that it requires up to seven days obtain results, which may not be practical for situations that demand rapid risk assessment (Boehm et al., 2009). The stain-based method is relatively rapid compared to the incubation method however, both methods requires highly skilled personnel to accurately distinguish between ova/larvae of different helminths. Due to the similarities in morphological characters of ova/larvae of *A. duodenale* and *A. caninum*, *N. americanus*, it is difficult to differentiate them into species (Cabaret et al., 2002; Verweij et al., 2007; Traub et al., 2008). In addition, the detection limit of both methods depends on the trained staff and detection sensitivity of a microscope that may not be sensitive enough (5% sensitivity) to detect low numbers of ova in a sample (Weber et al., 1991).

Specific detection of hookworm ova/larvae is essential to determine the accurate health risk associate with the use and reuse of wastewater as well as formulate an effective control measures. Developments in real-time polymerase chain reaction (PCR) assays enable rapid, sensitive and specific detection of various microbial pathogens in faecal samples (Verweij et al., 2007; ten Hove et al., 2009; Taniuchi, et al., 2011; Ahmed et al., 2015). PCR methods can detect pathogens in a one-step closed-tube reaction with much higher sensitivity and specificity by directly amplifying a specific gene from a target microorganism within 2-4 h (Botes et al., 2013; Schar et al., 2013), thus overcoming the limitations of the incubation and stain methods. However, there are a number of limitations that constrain the routine use of PCR based assays for the detection of hookworm ova in wastewater matrices. The two major limitations are concentration of hookworm ova from wastewater matrices and the potential

presence of PCR inhibitors (organic and inorganic compounds) in the DNA samples that known to inhibit PCR reactions (Toze, 1999; Shannon et al,. 2007). Generally hookworm ova are concentrated by sedimentation in conjunction with floatation and filtration depending on the turbidity of a sample (US EPA, 2003; Dryden et al., 2005; Goodman et al., 2007; Koompapong et al., 2009; Elom et al., 2012;). Sample processing time using these concentration methods can be lengthy. There is need for a rapid and simple method for the concentration of helminths ova from complex wastewater matrices that can be combined with PCR assay (Karkashan et al., 2014).

The main objective of this study was to investigate whether PCR method can be used for the rapid, sensitive and specific detection of hookworm ova in wastewater matrices. Canine hookworm (*Ancylostoma caninum*) was chosen as a representative hookworm to develop a PCR method for the detection of hookworm ova in wastewater matrices.

2. Methods

2.1. Rationale of using *A. caninum* as a representative hookworm

Human hookworm infection is less common in Australia and therefore, it was difficult to obtain large numbers of ova that were needed for the spiking experiment (see below). Therefore, *A. caninum* was chosen as a representative hookworm because of their high prevalence in dog feces in Australia. In addition, *A. caninum* ova have high morphological similarities with other hookworms of interests such as *A. duodenale* and deemed to be a suitable representative.

2.2. Real-time PCR positive control

A. caninum larvae were kindly donated by Associate Professor Dr. Rebecca Traub from the University of Melbourne, Melbourne, Australia for the development of the real-time PCR assay. DNA was extracted from the larvae using DNeasy Blood and Tissue® Kit (Qiagen,

Valencia, CA) according to the manufacturer's instructions. DNA was eluted in 100 µL AE buffer and stored at -80^{o}C freezer until further used.

2.3. Primer and probe design

Nucleotide sequences of the 5.8S rDNA of internal transcribe spacer (ITS) -1 region of *A. caninum* (NCBI accession no KC 755029.1) and *A. duodenale* (Accession no EU 344797.1) were obtained from GenBank. To identify the variation between the genes, the sequences were analysed using the NCBI Align Sequences Nucleotide Tool. A new set of forward primer DHF (5´-TTT GCT AAC GTG CAC TGA ATG-3´), reverse primer DHR (5´-GAA ACA CCG TTG TCA TAC TAG CC-3´) and probe DHP (FAM-5´-AAC TCG TTG TTG CTG CTG AA-BHQ1-3´) were designed and used to amplify a 101 base pair (bp) of the 5.8S rDNA gene of ITS-1 at 626-716 region of *A. caninum*.

2.4. Real-time PCR optimization and conditions

Genomic DNA from *A. caninum* larvae was used for the optimization of the real-time PCR assay. To ensure optimal performance of the PCR, primer concentrations ranging from 100 nM-400 nM and probe concentrations ranging from 300 nM-800 nM were titrated. The annealing temperature was optimized by performing a gradient analysis ranging from 55-61°C. The optimized real-time PCR amplifications were then performed in 25 µL reaction mixtures containing 12.5 µL iQ^{TM} Supermix (Bio-Rad Laboratories, Calif), 250 nM of each primer, 400 nM of probe, 3 µL of template DNA and UltraPure^{TM} DNase/RNase-free distilled water (Life Technologies, Australia). PCR cycling parameters were as follow: 95°C for 15 min, 95°C for 15 s, 59°C for 1 min. For each PCR assay, a positive (*A. caninum* DNA) and negative (UltraPure^{TM} water) controls were included.

2.5. Real-time PCR lower limit of detection (LLOD)

The PCR LLOD was determined using the stored genomic DNA extracted earlier from the *A. caninum* larvae. The DNA was quantified using a NanoDrop spectrophotometer (ND-1000,

NanoDrop Technology). Ten-fold serial dilutions (10^{-1} to 10^{-5}) of 5 ng of DNA were prepared in replicates (n = 6) and tested with PCR. The lowest quantity of DNA detected consistently in all replicate reactions was considered as the PCR LLOD.

2.6. Obtaining and enumerating the *A. caninum* ova

The *A. caninum* ova used in this study were collected from dog fecal samples that tested positive with microscopic examination at the School of Veterinary Science in University of Queensland, Gatton, Australia. *A. caninum* ova were isolated from approximately 20 gm of dog fecal samples using the floatation method (Bowman et al., 2003). After isolation, ova were preserved in 0.5% formalin and the numbers of were determined by microscopic observations (10x × 40x) using Sedgewick-Rafter Counting Chamber (Pyser-Sgi^{TM}) and enumerated in each grid in triplicates. The enumerated ova were stored at 4^{o}C and used for spiking experiments.

2.7. Sample limit of detection (SLOD)

To determine the SLOD, secondary treated wastewater, raw wastewater and sludge samples were collected from two wastewater treatment plants (WWTPs) "A" (27^{o}33'14.81"S; 152^{o}59'29.26" E) and "B" (27^{o}22'52.71"S; 153^{o}08'52.33"E)] located in Brisbane, Qld, Australia. Fifteen litres of secondary treated wastewater and raw wastewater were collected from each WWTP in sterile 20 L polypropylene carboys. Secondary treated wastewater was collected before chlorination whereas raw wastewater sample was collected from the primary influent. Dewater sludge samples were also collected from the belt process from each WWTP in 500 mL sterile polyethylene Zip Lock^{TM} bags. All samples were transported on ice to the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Land and Water laboratory, Dutton Park, Queensland. Approximately 4.0×10^{3} of *A. caninum* ova were added to 1 L tap water (control), secondary treated wastewater and raw wastewater samples. Ten-fold serial dilutions (10^{-1} to 10^{-4}) were made for each sample. For the sludge experiment,

10-fold serial dilutions (10^{-1} to 10^{-4}) of 4.0×10^{3} ova were made and added to approximately 4 gm of samples. All samples were processed in triplicate according to the concentration method described in section 2.8. A method blank of unspiked sample was included for each batch of tap water, secondary treated and raw wastewater samples to check for cross contamination during sample processing. Before adding the ova, the tap water, secondary treated wastewater, raw wastewater and sludge samples were screened for the presence of *A. caninum* rDNA gene by using the newly developed PCR method to obtain information on any background levels if any.

2.8. Sample concentration and DNA extraction

Tap water and secondary treated wastewater samples were filtered through 8 μm, 90 mm polycarbonate filters (Merck Millipore, Billerica, Massachusetts, USA) using glass funnels attached to a vacuum pump. The ova trapped were washed from the filters into a 50 mL polypropylene tube with 25mL phosphate buffer saline (PBS). The ova were pelleted from the PBS suspension by centrifuging at 800 *g* for 15 min. First, attempts to process raw wastewater samples (1L) with membrane filtration method failed due to rapid clogging of the filters by materials suspended in the wastewater. Subsequently, a centrifugation and flotation approach was used to concentrate the ova from raw wastewater samples. Raw wastewater samples (1 L) were transferred into 700 mL centrifuge containers (Beckman Coulter Inc), and centrifuged at 5,200 *g* for 30 min. The resulting supernatant was removed and pellet was resuspended in 20 mL MilliQ water and transferred to a 50 mL polypropylene centrifuge tube, and centrifuged for 10 min to obtain a pellet. Ova were then separated from the pellet with the $MgSO_4$ (specific gravity 1.2) floatation method outlined previously by Bowman et al. (2003). In brief, the pellet was suspended in 45 mL of $MgSO_4$ solution by vortexing in a centrifuge tube. The suspension was then centrifuged at 800 *g* for 3 min and the supernatant

was transferred into a 15 mL polypropylene tube, and further centrifuged at 800 g for 10 min to obtain a pellet.

A similar approach was also followed for the sludge. In brief, sludge samples (5 gm wet weight) were placed into a 50 mL polypropylene and 45 mL of $MgSO_4$ solution was added. The tube was then centrifuged at 800 *g* for 3 min and the supernatant was transferred into a 15 mL polypropylene tube, and further centrifuged at 800 g for 10 min to obtain a pellet.

DNA from the concentrated hookworm ova in tap water and secondary treated wastewater (WWTP-A) was extracted using DNeasy Blood and Tissue Kit (Qiagen) with slight modification. In brief, pellets from the centrifugation step were by mixed with 180 µL of lysis buffer ATL followed by five freeze-thaw cycles of 10 min each at -80ºC and 95ºC. Due to the presence of high suspended solid contents and observed PCR inhibition in the secondary treated wastewater from WWTP-A, MO Bio Power Soil® DNA Kit (Mo Bio, Carlsbad, CA) was used to extract DNA for all further samples from WWTP-A and WWTP-B. In this case, pellets from the centrifugation step were by mixed with 60 µL of lysis buffer C1 followed by five freeze-thaw cycles of 10 min each. A reagent blank was also included during DNA extraction.

2.9. Application of the novel real-time PCR method

To determine the application of the method, tap water, secondary treated wastewater, raw wastewater and sludge samples were also collected from two different WWTPs in different time intervals. Sample concentration and DNA extraction were conducted as described above but without spiking the canine hookworm ova.

2.10. PCR inhibition test

A sketa22 real-time PCR assay with previously published primers and probe was used to determine the presence of inhibitors in the DNA samples extracted from tap water, secondary treated wastewater, raw wastewater and sludge samples (Haugland et al., 2005). All DNA

samples were spiked with 10 pg of *O. keta* DNA (Ahmed et al., 2015). The threshold cycle (C_T) values of the *O. keta* contaminated DNA samples were compared to equivalent quantities of *O. keta* DNA suspended in UltraPure™ water (Life Technologies). Where inhibition was detected, the DNA extracts were diluted 10 fold and retested. The sketa22 PCR assay was performed in 25 µL reaction mixtures using iQ™ Supermix (Bio-Raid Laboratories). The sketa22 PCR assay mixture contained 12.5 µL of Supermix, 300 nM of each primer, 400 nM of probe, 10 pg of *O. keta* DNA and 3 µL of template DNA sample.

2.11. Statistical analysis

GraphPad Prism 6 (GraphPad Software) was used to conduct the statistical analysis. A one-way ANOVA was performed to determine the differences between the C_T values obtained for *O. keta* DNA suspended in UltraPure™ water and *O. keta* contaminated DNA samples extracted from tap water and wastewater matrices samples. ANOVA was also used to determine whether the C_T values obtained for spiked and non spiked secondary treated wastewater, raw wastewater and sludge samples varied significantly within and between WWTPs as well as tap water samples.

3. Results

3.1. Real-time PCR optimisation and lower limit of detection (LLOD)

The specificity of the newly designed primers was checked using NCBI Megablast. The results of the BLAST search showed that the sequences of selected primers matched with the 5.8s rDNA of ITS-1 region of *A. caninum*. Among the series of primer and probe concentrations tested, 250 nM of each primer and 400 nM of probe provided the earliest C_T value (22.2 ± 0.07) at the annealing temperature of 59°C. The LLOD of the real-time PCR assay was performed using genomic DNA from *A. caninum* larvae. The PCR assay was able to detect *A. caninum* DNA up to dilution 10^{-4} (equivalent to 500 fg) (Table 1).

3.2. PCR inhibitors

The mean C_T value and standard deviation for the *O. keta* spiked UltraPure™ water was 27.8 ± 0.36. The mean C_T values and standard deviations values for *O. keta* spiked tap water, secondary treated wastewater, raw wastewater and sludge DNA samples were similar to *O. keta* spiked UltraPure™ water (Table 2). The secondary treated wastewater DNA samples from WWTP-A and sludge DNA samples from both WWTPs did not amplify *O. Keta* DNA, indicating the presence of PCR inhibitors. These DNA samples were serially diluted to reduce any PCR inhibitors and re-analysed. The mean C_T values and standard deviations value for *O. keta* spiked diluted (10 fold) secondary treated wastewater and sludge samples were 27.9 ± 0.21 and 28.5 ± 0.54 (WWTP-A) and 28.1 ± 0.11 (WWTP-B), respectively. An ANOVA analysis on the C_T values obtained for *O. keta* spiked UltraPure™ water and all the undiluted DNA samples that were free of PCR inhibitors as well as the 10 fold diluted samples showed that there was no significant ($P > 0.05$) difference suggesting the absence of PCR inhibitors. Based on these results, all the samples without PCR inhibition (undiluted and 10-fold diluted samples) were used for the PCR assays.

3.3. Sample limit of detection (SLOD) of *A. caninum* ova spiked wastewater and sludge samples

The real-time PCR method indicated that, the background wastewater samples used for seeding experiments were free from *A. caninum* rDNA. The method was able to detect *A. caninum* ova at a dilution of 10^{-4} (< 1 ova) for ova spiked tap water samples (Table 3). Similar results were also obtained for ova spiked secondary treated wastewater from both WWTPs. The SLOD of *A. caninum* ova for the ova spiked raw wastewater and sludge samples from both WWTPs was at dilution of 10^{-3} (4 ova) indicating lower detection of *A. caninum* ova in these matrices compared to secondary treated wastewater.

The range of mean C_T values obtained for the *A. caninum* ova spiked into tap water samples was < secondary treated wastewater < raw wastewater < sludge samples from both

WWTPs (Figure 1a and b). The C_T value of tap water samples were significantly ($P < 0.05$) different than from secondary treated wastewater from WWTP-A and raw wastewater and sludge samples from both WWTPs. Significant differences were also observed for the secondary treated wastewater with raw wastewater and sludge samples for both WWTPs. However, the C_T values of raw wastewater and sludge did not differ significantly ($P > 0.05$) from each other for both WWTPs. The C_T values of the secondary treated wastewater, raw wastewater and sludge samples (for WWTP-A) came later than those obtained for WWTP-B. However, the differences were not statistically significant ($P > 0.05$).

3.4. Application of the real-time PCR method to detect *A. caninum* ova in unspiked samples

Among the tap water ($n = 3$), secondary treated wastewater ($n = 12$), raw wastewater ($n = 18$) and sludge ($n = 6$) samples tested, *A. caninum* DNA were detected in secondary treated wastewater (50%), raw wastewater (38%) and sludge (33.3%) samples (Figure 2). The secondary treated wastewater had the higher C_T values ($C_T = 36.9 \pm 0.80$) followed by raw wastewater ($C_T = 35.6 \pm 2.43$) and sludge samples ($C_T = 34.9 \pm 2.43$).

4. Discussion

The application of PCR-based methods has generated interest for the direct monitoring of parasites in fecal samples (Verweij et al., 2007; Yong et al., 2007; Traub et al., 2008; Ngui et al., 2012; Schar et al., 2013). PCR methods are rapid and can be used to detect specific parasites of interest with high sensitivity. Detection of helminths ova from wastewater matrices however, require isolation and concentration of the ova, which is often challenging due to the presence of high suspend solids and PCR inhibitors. In this study, a probe based real-time PCR method was developed for the rapid, sensitive and specific detection of canine hookworm (*A. caninum)* ova from wastewater matrices.

The sensitivity of the real-time PCR assay was thoroughly tested by amplifying known concentration of genomic DNA obtained from *A. caninum* larvae. The PCR LLOD of the newly developed assay was determined to be 500 fg of genomic DNA for all replicate samples which is similar or one order of magnitude lower than the LLOD values reported in previous studies (Thaenkham et al., 2007; Traub et al., 2009; Rahman et al., 2011; Taniuchi et al., 2011; Ngui et al., 2012).

We also determined for the effects of PCR inhibitors on the detection of *A. caninum* ova in all wastewater matrices. Our results indicated that DNA samples from the secondary treated wastewater (from WWTP-A) had PCR inhibitors. DNeasy Blood and Tissue Kit was used to extract DNA from these samples, and it is possible that the kit was unable to remove the inhibitors effectively. In view of this, MO Bio Power Soil® DNA Kit was used for DNA isolation from the remaining wastewater and sludge samples. The main advantage of the MO Bio Power Soil® DNA Kit over the DNeasy Blood and Tissue Kit is the ability to remove humic substances and other inhibitors. This is supported by the fact that secondary treated wastewater DNA samples from WWTP-B and raw wastewater samples from both WWTPs had no PCR inhibitors. However, sludge DNA samples from both WWTPs indicated the presence of PCR inhibitors despite the use of MO Bio Power Soil® DNA Kit. This could be due to the fact that the concentrations of PCR inhibitors in sludge samples could be higher than raw wastewater samples (Schrader et al., 2012).

Ten-fold serial dilution of DNA is commonly applied strategy in environmental, clinical, food samples to overcome PCR inhibition (Drosten et al., 2002; Audemard et al., 2004; Van Doorn et al., 2009). Our results indicated that a 10 fold-dilution of samples with PCR inhibition was adequate to relieve the inhibitors. Based on our data, we recommend that DNA samples extracted from large volumes of wastewater matrices or complex environmental

samples should be checked for the presence of PCR inhibitors prior to use for PCR amplification.

The PCR method used in this study was capable of detecting *A. caninum* ova in a range of <1 to 4 across all wastewater matrices from both WWTPs. These results were consistent for all dilutions across the both WWTPs. The earlier C_T values for the secondary treated wastewater indicated the better recovery of ova from treated wastewater than raw wastewater and sludge samples. This could be attributed to low suspended solid content of secondary treated wastewater making it possible to pass through the membrane using the membrane filtration method. It has been reported that ova were better retained on the membranes during filtering of water samples compare to a potential loss of ova during floatation (Nieminski et al., 1995; Ferguson et al., 2004). Because of the high solid contents and turbidity, membrane filtration method was not suitable for processing raw wastewater and sludge samples. In view of this, we chose to use the flotation technique to concentrate ova from raw wastewater and sludge samples. Another important point to consider is that the flotation technique involves multiple steps of centrifugation, flotation and concentrations with potential loss of ova in each step compared to the membrane filtration method, which involves a single step for recovering the ova directly from the filters.

A significant challenge still remains to develop a rapid and effective ova recovery method for helminths from raw wastewater and sludge. This requires further research to develop and validate new rapid concentration method. For instance, hollow-fibre ultrafiltration method has been shown to recover up to 83% *Giardia* and *Cryptosporidium* oocysts from environmental waters (Kuhn and Oshima, 2002; Hill et al., 2009). It is therefore, recommended that concentration methods such as hollow-fibre ultrafiltration needs to be compared with membrane filtration and flotation methods in terms of method recovery efficiency.

Our newly developed real-time PCR method was validated by testing of tap water, secondary treated wastewater, raw wastewater and sludge samples from two different WWTPs. The method was able to detect low levels of *A. caninum* DNA from all three matrices. The earlier C_T values (Figure 2) obtained for the sludge and raw wastewater samples than secondary treated wastewater suggests the presence of relatively more DNA in the sludge and raw wastewater samples despite the potential poor recovery of the floatation method. This indicates that the method developed for the detection of hookworm ova from the wastewater matrices could also be adapted to detect other pathogenic helminth ova such as *A. duodenale, N. americanus, Ascaris lumbricoides* from wastewater matrices and other environmental samples.

As with other methods, a major limitation of our method is its inability to distinguish between viable and nonviable *A. caninum* ova from wastewater matrices. It can therefore, not be ruled out that our method may have detected DNA from nonviable *A. caninum* ova in the unspiked wastewater matrices. The application of EMA/PMA should be investigated to discriminate nonviable ova from wastewater matrices, as it has been reported that the EMA/PMA combined with PCR method can eliminate potentially non-viable DNA during PCR amplification (Nocker, et al., 2006; Nocker, et al., 2007; Pan and Bredit, 2007; Loozen et al. 2011). Currently, we are developing a PMA-PCR method for the detection of viable hookworm ova in wastewater matrices.

5. Conclusions

- We developed and validated a specific and sensitive real-time PCR method that can detect *A. caninum* ova in wastewater matrices. This method is rapid and can produce result with 6-8 h compared to incubation and stain methods that could take up to 2 weeks and lack specificity in distinguishing ova/larvae to the species level.

- For ova spiked secondary treated wastewater, the SLOD of the method was < 1 ova while this value for the ova spiked raw wastewater and sludge samples were 4 ova indicating the high sensitivity of the method.
- The method can also be adopted to detect other pathogenic helminth ova such as *A. duodenale, N. americanus* and *A. lumbricoides* from wastewater matrices.
- Further studies would be required to discriminate non-viable ova hookworm ova from the wastewater matrices and environmental samples.

Acknowledgement

The authors would like to appreciate financial support from the Water Corporation, WA and the Commonwealth Scientific and Industry Research Organisation (CSIRO), Land and Water Flagship. The authors are thankful to Associate Professor Rebecca Traub, University of Melbourne and Lyn Knott, Laboratory Manager Faculty of Veterinary Science, University of Queensland, Australia for providing *A. caninum* positive controls and *A. caninum* positive dog fecal samples respectively. We also thank, Queensland Urban Utilities for providing access to the wastewater treatment plants for collecting samples.

Reference

Abaidoo, R. C., Keraita, B., Drechsel, P., Dissanayake, P., Maxwell. A. S., 2010. Soil and crop contamination through wastewater irrigation and options for risk reduction in developing countries. P. Dion (ed.), Soil Biology and Agriculture in the Tropics, Soil Biology 21. Pages 275-297.

Ahmed, W., Gyawali, P., Toze S., 2015. Quantitative PCR measurements of Escherichai coli including shiga-toxin-producing E. coli (STEC) in animal feces and environmental waters. Envron Sci Technol. 49 (5), 3084-3090.

Audemard, C., Reece, K. S., Burreson, E. M., 2004. Real-Time PCR for Detection and Quantification of the Protistan Parasite *Perkinsus marinus* in Environmental Waters. Appl Environ Microbiol. 70 (11), 6611-6618.

Bethony, J., Brooker, S., Albonico, M., Geiger, S. M., Loukas, A., Diemert, D., Hotez, P.J., 2006. Soil-transmitted helminth infections: ascariasis, trichuriasis, and hookworm. Lancet. 367 (9521), 1521-1532.

Boehm, A. B., Ashbolt, N. J., Colford, J. M., Dunbar, L. E., Fleming, L. E., Gold, M. A., Hansel, J. A., Hunter, P. R., Ichida, A. M., McGee, C. D., Soller, J. A., Weisberg, S. B., 2009. A sea change ahead for recreational water quality criteria. J Water Health. 7 (1), 9-20.

Botes, M., De Kwaadsteniet, M., Cloete, T. E., 2013. Application of quantitative PCR for the detection of microorganisms in water. Anal Bioanal Chem. 405 (1), 91-108.

Bowman, D. D., Little, M. D., Reimers, R. S., 2003. Precision and accuracy of an assay for detecting *Ascaris* eggs in various biosolid matrices. Water Res. 37 (9), 2063-2072.

Brookers, S., 2010. Estimating the global distribution and disease burden of intestinal nematode infections: adding up the numbers. a review. Int J Parasitol. 40 (10), 1137-1144.

Brooker, S., Clements, A. C., Bundy, D. A., 2006. Global Epidemiology, Ecology and Control of Soil-Transmitted Helminth Infections. Adv Parasitol. 62, 221-261.

Brooker, S., Hotez, P. J., Bundy, D. A., 2008. Hookworm-related anaemia among pregnant women: a systematic review. PLoS Negl Trop Dis. 2 (9), e291.

Cabaret, J., Geerts, S., Madeline, M., Ballandonne, C., Barbier, D., 2002. The use of urban sewage sludge on pastures: the cysticercosis threat. Vet Res. 33 (5), 575-597.

Carr, R., 2005. WHO guidelines for safe wastewater use-more than just numbers. Irrig Drain. 54, S103-S111.

de Victorica, J., Galvan, M., 2003. Preliminary testing of a rapid coupled methodology for quantitation viability determination of helminth eggs in raw and treated wastewater. Water Res. 37 (6), 1278-1287.

Do, T. T., Molbak, K., Phung, D. C., Dalsgaard, A., 2007. Helminth infections among people using wastewater and human excreta in peri-urban agriculture and aquaculture in Hanoi, Vietnam. Trop Med Int Health. 12 (sup 2), 82-90.

Drosten, C., Panning, M., Guenther, S., Schmitz, H., 2002. False-negative results of PCR assay with plasma of patients with severe viral hemorrhagic fever. J Clin Microbiol. 40 (11), 4394-4395.

Dryden, M. W., Payne, P. A., Ridley, R., Smith, V., 2005. Comparison of common fecal flotation techniques for the recovery of parasite eggs and oocysts. Vet Ther. 6(1), 15-28.

Elom, M. O., Eze, U. A., Nworie, A., Akpotomi, I. O., 2012. Prevalence of geohelminths on edible fruits and vegetables cultivated in rural villages of Ebonyi State, South East Nigeria. Am J Food Nutr. 2(3), 58-64.

Ensink, J. H., vander Hoek, W., Mukhtar, M.,Tahir, Z., Amerasinghe, F. P., 2005. High risk of hookworm infection among wastewater farmers in Pakistan. Trans R Soc Trop Med Hyg. 99 (11), 809-818.

Ferguson, C., Kaucner, C., Krogh, M., Deere, D., Warnecke, M., 2004. Comparison of methods for the concentration of *Cryptosporidium* oocysts and *Giardia* cysts from raw waters. Can J Microbiol. 50 (9), 675-682.

Goodman, D., Haji, H. J., Bickle, Q. D., Stoltzfus, R. J., Tielsch, J. M., Ramsan, M., Savioli, L., Albonico, M., 2007. A comparison of methods for detecting the eggs of Ascaris, Trichuris, and hookworm in infant stool, and the epidemiology of infection in Zanzibari infants. Am J Trop Med Hyg. 76(4), 725-731.

Gyawali, P., 2012. Parasitic Diseases of Indigenous Community (Kumal) in Nepal. Nep J Sci and Tec. 13 (2), 175-178.

Hanjra, M. A., Blackwell, J., Carr, G., Zhang, F., Jackson, T. M., 2012. Wastewater irrigation and environmental health: implications for water governance and public policy. Int J Hyg Environ Health. 215 (3), 255-269.

Haugland, R. A., Siefring, S. C., Wymer, L. J., Brenner, K. P., Dufour, A. P., 2005. Comparison of *Enterococcus* measurements in freshwater at two recreational beaches by quantitative polymerase chain reaction and membrane filter culture analysis. Water Res. 39 (4), 559-568.

Hill, V. R., Polaczyk, A. L., Kahler, A. M., Cromeans, T. L., Hahn, D., Amburgey, J. E., 2009. Comparison of hollow-fiber ultrafiltration to the USEPA VIRADEL technique and USEPA method 1623. J Environ Qual. 38 (2), 822-825.

Hotez, P. J., Alvarado, M., Basanez, M. G., Bolliger, I., Bourne, R., Boussinesq, M., Brooker, S. J., Brown, A. S., Buckle, G., Budke, C. M., Carabin, H., Coffeng, L. E., Fevre, E. M., Furst, T., Halasa, Y. A., Asrasaria, R., Johns, N. E., Keiser, J., King, C. H., Lozano, R., Murdoch, M. E., O'hanlon, S., Pion, S. D., Pullan, R. L., Ramaiah, K. D., Roberts, T., Shepard, D. S., Smith, J. L., Stolk, W. A., Undurraga, E. A., Utzinger, J., Wang, M., Murray, C. J., Naghavi, M., 2014. The Global Burden of Disease Study 2010: Interpretation and Implications for the Neglected Tropical Diseases. PLoS Negl Trop Dis. 8 (7), e2865.

Hotez, P. J., Bethony, J., Bottazzi, M. E., Brooker, S., Buss, P., 2005. Hookworm: "The Great Infection of Mankind". PLoS Med. 2 (3), e67.

Karkashan, A., Khallaf, B., Morris, J., Thurbon, N., Rouch, D., Smith, S. R., Deighton, M., 2014. Comparison of methodologies for enumerating and detecting the viability of Ascaris eggs in sewage sludge by standard incubation-microscopy, the BacLight live/Dead viability assay and other vital dyes. Water Res. DOI: 10.1016/j.waters.2014.10003.

Kelessidis, A., Stasinakis, A. S., 2012. Comparative study of the methods used for treatment and final disposal of sewage sludge in European countries. Waste Manag. 32 (6), 1186-1195.

Knopp, S., Steinmann, P., Keiser, J., Utzinger, J., 2012. Nematode infections: soil-transmitted helminths and *Trichinella*. Infect Dis Clin North Am. 26 (2), 341-358.

Koompapong, K., Sutthikornchai, C., Sukthana, Y., 2009. Cryptosporidium oocysts detection in water samples: floatation technique enhanced with immunofluorescence is as effective as immunomagnetic separation method. Korean J Parasitol. 47(4), 353-357.

Kuhn, R. C., Oshima, K. H., 2002. Hollow-fiber ultrafiltration of *Cryptosporidium parvum* oocysts from a wide variety of 10-L surface water samples. Can J Microbiol. 48 (6), 542-549.

493 Loozen, G., Boon, N., Pauwels, M., Quirynen, M., Teughels, W., 2011. Live/dead real-time
494 polymerase chain reaction to assess new therapies against dental plaque-related
495 pathologies. Mole Oral Microbiol 26**:**253-261.
496 Ngui, R., Lim ,Y. A. L., Chuk, K. H., 2012. Rapid detection and identification of human
497 hookworm infections through high resolution melting (HRM) analysis. PloS One. 7
498 (7)**,** e41996.
499 Nieminski, E. C., Schaefer, F., Ongerth, J. E., 1995. Comparison of two methods for
500 detection of *Giardia* cysts and *Cryptosporidium* oocysts in water. Appl Environ
501 Microbiol. 61 (5), 1714-1719.
502 Nocker, A., Cheung, C. Y., Camper, A. K., 2006. Comparison of propidium monoazide with
503 ethidium monoazide for differentiation of live vs. dead bacteria by selective removal
504 of DNA from dead cells. J Microbiol Methods 67**:**310-320.
505 Nocker, A., Sossa, K. E., Camper, A. K., 2007. Molecular monitoring of disinfection efficacy
506 using propidium monoazide in combination with quantitative PCR. J Microbiol
507 Methods 70**:**252-260.
508 Pan, Y., Breidt, F., 2007. Enumeration of viable Listeria monocytogenes cells by real-time
509 PCR with propidium monoazide and ethidium monoazide in the presence of dead
510 cells. Appl Environ Microbiol 73**:**8028-8031.
511 Pritchard, D. L., Penney, N., McLaughlin, M. J., Rigby, H., Schwarz, K., 2010. Land
512 application of sewage sludge (biosolids) in Australia: risks to the environment and
513 food crops. Water Sci Technol. 62 (1), 48-57.
514 Rahman, S. M., Bae, Y. M., Hong, S. T., Choi, M. H., 2011. Early detection and estimation
515 of infection burden by real-time PCR in rats experimentally infected with *Clonorchis*
516 *sinensis*. Parasitol Res. 109 (2), 297-303.
517 Schar, F., Odermatt, P., Khieu, V., Panning, M., Duong, S., Muth, S., Marti, H., Kramme, S.,
518 2013. Evaluation of real-time PCR for *Strongyloides stercoralis* and hookworm as
519 diagnostic tool in asymptomatic schoolchildren in Cambodia. Acta Trop. 126 (2), 89-
520 92.
521 Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012. PCR inhibitors-occurrence,
522 properties and removal. J Appl Microbiol. 113 (5), 1014-1026.
523 Shannon, K., Lee, D. Y., Trevors, J., Beaudette, L., 2007. Application of real-time
524 quantitative PCR for the detection of selected bacterial pathogens during municipal
525 wastewater treatment. Sci total Environ. 382 (1), 121-129.
526 Sharafi, K., Fazlzadehdavil, M., Pirsaheb, M., Derayat, J., Hazrati, S., 2012. The comparison
527 of parasite eggs and protozoan cysts of urban raw wastewater and efficiency of
528 various wastewater treatment systems to remove them. Ecological Engin. 44, 244-
529 248.
530 Sidhu, J. P. S., Toze, S., 2009. Human pathogens and their indicators in biosolids: A litreature
531 review. Environ Int. 35 (1), 187-201.
532 Taniuchi, M., Verweij, J. J., Noor, Z., Sobuz, S. U., Lieshout, L., Petri, W. A., Haque, R.,
533 Houpt, E. R., 2011. High throughput multiplex PCR and probe-based detection with
534 Luminex beads for seven intestinal parasites. Am J Trop Med Hyg. 84 (2), 332-337.
535 tenHove, R. J., vanEsbroeck, M., verVoort, T., vanDenende, J., vanLieshout, L., Verweij, J.
536 J., 2009. Molecular diagnostics of intestinal parasites in returning travellers. Eur J
537 Clin Microbiol Infect Dis. 28 (9), 1045-1053.
538 Thaenkham, U., Visetsuk, K., Dung, D. T., Waikagul, J., 2007. Discrimination of
539 *Opisthorchis viverrini* from *Haplorchis taichui* using COI sequence marker. Acta
540 Trop. 103 (1), 26-32.
541 Toze, S., 1999. PCR and the Detection of Microbial Pathogens in Water and Wastewater.
542 Water Res. 33 (17), 3545-3556.

Toze, S., Sidhu, J., 2011. Biosolids:Human Health Impacts. Nriaju, J. (ed.). Encyclopedia of Environ Health. Elsevier, pp. 394-402

Traub, R. J., Inpankaew, T., Sutthikornchai, C., Sukthana, Y., Thompson, R. C., 2008. PCR-based coprodiagnostic tools reveal dogs as reservoirs of zoonotic ancylostomiasis caused by *Ancylostoma ceylanicum* in temple communities in Bangkok. Vet Parasitol. 155 (1-2), 67-73.

Traub, R. J., Macaravas, J., Mungthin, M., Leelayoova, S., Cribb, T., Murrell, K. D., Thompson, R. A., 2009. A new PCR-based approach indicates the range of *Clonorchis sinensis* now extends to Central Thailand. PLoS Negl Trop Dis. 3 (1), e367.

United State Environment protection Agency, 2003. Control of pathogens and vector attraction in sewage sludge. USEPA Environmental Regulations and Technology. Office of Research and Development, EPA/625/R-92/013. Washington DC

vanDoorn, R., Klerks, M., vanGent-Pelzer, M., Speksnijder, A., Kowalchuk, G., Schoen, C., 2009. Accurate quantification of microorganisms in PCR-inhibiting environmental DNA extracts by a novel internal amplification control approach using Biotrove OpenArrays. Appl Environ Microbiol. 75 (22), 7253-7260.

Verweij, J. J., Brienen, E. A. T., Ziem, J., Yelifari, L., Polderman, A. M., venLieshout, L., 2007. Simultaneous Detection and Quantification of *Ancylostoma duodenale, Necator americanus*, and *Oesophagostomum bifurcum* in Fecal Samples Using Multiplex Real-Time PCR. Am J Trop Med Hyg. 77 (4), 685-690.

Weber, R., Bryan, R. T., Bishop, H. S., Wahlquist, S. P., Sullivan, J. J., Juranek, D. D., 1991. Threshold of detection of *Cryptosporidium* oocysts in human stool specimens: evidence for low sensitivity of current diagnostic methods. J Clin Microbiol. 29 (7), 1323-1327.

Wen, Q., Tutuka, C., Keegan, A., Jin, B., 2009. Fate of pathogenic microorganisms and indicators in secondary activated sludge wastewater treatment plants. J Environ Manag. 90 (3), 1442-1447.

World Health Organisation, 2006. Guidelines For the safe use of Wastewater, Excreta and Greywater in agriculture, Vol. 2 and Vol. 4. WHO Library Cataloguing-in-Publication Data, p.213.

World Health Organisation, 2012. Soil-transmitted helminthiases: eliminating soil-transmitted helminthiases as a public health problem in children: progress report 2001-2010 and strategic plan 2011-2020.

Yong, T. S., Lee, J. H., Sim, S., Lee, J., Min, D. Y., Chai, J. Y., Eom, K. S., Sohn, W. M., Lee, S. H., Rim, H. J., 2007. Differential diagnosis of *Trichostrongylus* and hookworm eggs via PCR using ITS-1 sequence. Korean J Parasitol. 45 (1), 69-74.

Table 1: Real-time PCR lower limit of detection (LLOD) of serially diluted *A. caninum* genomic DNA (n = 6)

DNA dilutions	Positive samples (%)	Mean ± SD of C_T values
10^{-1}	100	25.6 ± 0.04
10^{-2}	100	29.1 ± 0.04
10^{-3}	100	32.4 ± 0.09
10^{-4}	100	36.6 ± 0.10
10^{-5}	0	ND

C_T: Threshold cycle
ND: Real-time PCR not detected
SD: Standard deviation

Table 2: Sketa22 PCR assay for the evaluation of PCR inhibition in *A. caninum* ova spiked tap water (n = 12), secondary treated wastewater (n = 12), raw wastewater (n = 12) and sludge (n = 12) DNA samples as opposed to salmon testes DNA (n = 10 pg) spiked UltraPure™ DNase/RNase-free distilled water samples

Wastewater treatment plants	Sample types	Mean ± SD of C_T values	
		Undiluted DNA	Diluted DNA (10^{-1})
WWTP-A	Secondary treated wastewater[a]	NPA	27.9 ± 0.21
	Raw Wastewater[b]	27.7 ± 0.45	N/A
	Sludge[b]	NPA	28.5 ± 0.54
WWTP-B	Secondary treated wastewater[b]	27.5 ± 0.30	N/A
	Raw Wastewater[b]	27.5 ± 0.23	N/A
	Sludge[b]	NPA	28.1 ± 0.11
	Tap water[a]	28.5 ± 0.12	N/A
	UltraPure™ DNase/RNase-free distilled water	27.8 ± 0.36	N/A

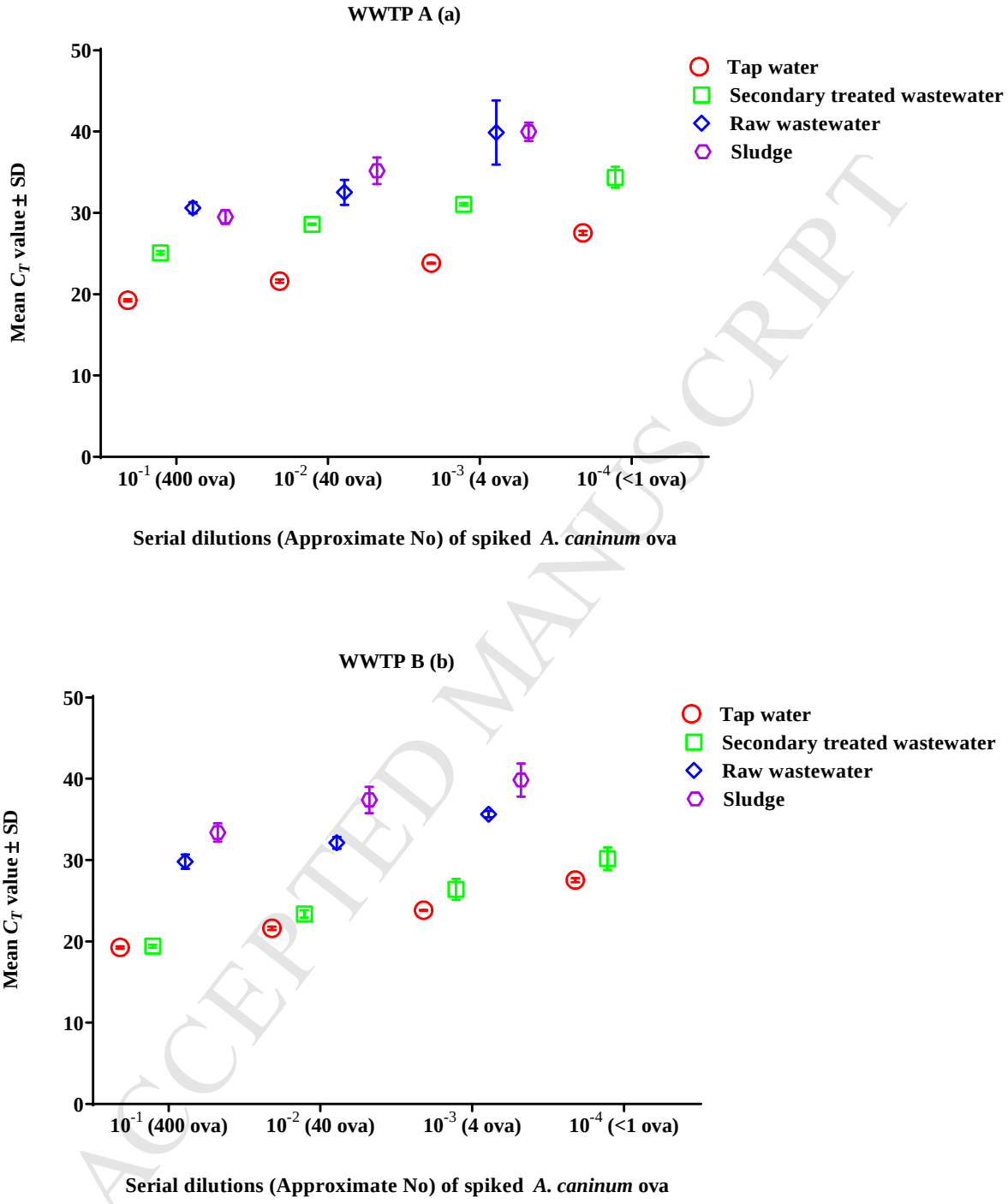
[a] :DNeasy Blood and Tissue Kit
[b]:MO Bio Power Soil® DNA Kit
NPA: No PCR amplification
N/A: Not applicable
C_T: Threshold cycle
SD: Standard deviation

Table 3: Method limit of detection (MLOD) of real-time PCR assay for detection of *A. caninum* ova in spiked tap water (n = 3), secondary treated wastewater (n = 3), raw wastewater (n = 3) and sludge (n = 3) samples

Source of samples	Wastewater matrices	Triplicate PCR results at the dilutions tested			
		10^{-1} (400 ova)	10^{-2} (40 ova)	10^{-3} (4 ova)	10^{-4} (< 1 ova)
CSIRO Lab	Tap water	+	+	+	+
WWTP-A	Secondary treated wastewater	+	+	+	+
	Raw wastewater	+	+	+	-
	Sludge	+	+	+	-
WWTP-B	Secondary treated wastewater	+	+	+	+
	Raw wastewater	+	+	+	-
	Sludge	+	+	+	-

+: Real-time positive results
-: Real-time negative results

665



666

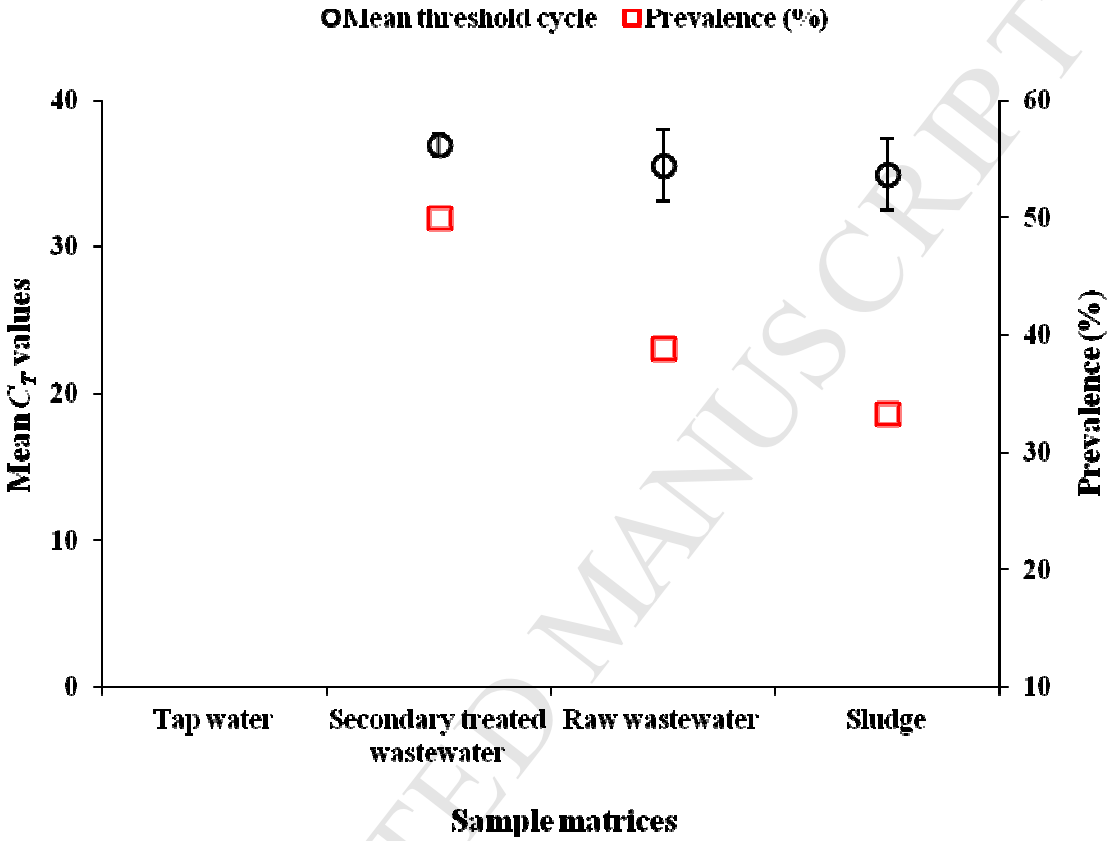
667

668 **Figure 1:** Mean and standard deviation of C_T value of serially diluted *A. caninum* ova spiked
669 DNA into secondary treated wastewater, raw wastewater and sludge samples from
670 wastewater treatment plants A (a) and B (b) compared to tap water.

671

672

673



674

675 **Figure 2:** Prevalence of *A. caninum* in unspiked tap water (*n* = 3), secondary treated
676 wastewater (*n* = 12), raw wastewater (*n* = 18) and sludge (*n* = 6) samples collected from
677 different wastewater treatment plants (WWTPs).

Highlights:

- Rapid and sensitive detection of hookworm ova from wastewater is paramount.
- We developed a novel PCR method for the detection of hookworm ova in wastewater.
- The sensitivity of the newly developed PCR method is one to four ovum in wastewater.
- The method can be used to detect ova of various helminths in wastewater matrices.